

# Evolution and Origins of Sperm Nuclear Basic Proteins

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## ABSTRACT

Although the DNA-binding, basic nuclear proteins of sperm (SNBPs) are highly diverse, constraints on their diversity in particular taxa suggest that they may play an adaptive role in such taxa and are not merely randomly distributed. For example, in the subphylum Vertebrata, internally fertilizing tetrapods have arginine-rich protamines or keratinous protamines that condense sperm chromatin to a greater extent than in externally fertilizing frogs or fish having sperm histones. In such taxa "R-CLUES," relatively constant largest units of evolutionary similarity, are broader phylogenetically for mammals (for example, infraclass Eutheria) than for frogs (for example, genus *Bufo*). The evolution of SNBPs appears to be saltatory rather than continuous. Consequently, the phylogenetic breadth of R-CLUES may assist in the classification of particular taxa where SNBPs are either protamine-like or histone-like amongst particular genera of frogs (*Xenopus* versus *Silurana*) or subfamilies/families of fish (Salmoninae versus Sparidae). SUBIRANA & COLOM (1987), AUSIO *et al.* (1987), and CHIVA *et al.* (1991) have proposed that protamines in molluscs may have evolved from very lysine-rich H1 histones. This is supported by the presence of protamine-like SNBPs with trypsin-resistant cores in cnidarian sperm. The presence of protamine-like SNBPs in lower plants may be a case of convergent evolution. Highly condensed chromosomes in the eukaryotic dinoflagellate *Cryptocodinium cohnii* and the highly condensed nucleoid in the bacterium *Chlamydia trachomatis*, both have lysine-rich basic proteins similar to portions of histones H5 or H1, respectively. This suggests possible additional cases of convergent evolution amongst basic proteins of condensed chromatin preceding the origin of the primitive type of sperm in metazoans.

## RÉSUMÉ

### Évolution et origines des protéines basiques nucléaires des spermatozoïdes

Bien que les protéines nucléaires basiques liées à l'ADN des spermatozoïdes (PNBS) soient extrêmement diversifiées, les contraintes pesant sur leur diversité dans des taxons particuliers suggèrent qu'elles pourraient jouer un rôle adaptatif dans de tels taxons et qu'elles ne sont pas simplement distribuées au hasard. Par exemple, dans le sous-embranchement Vertebrata, les Tétrapodes à fécondation interne ont des protamines riches en arginine ou des protamines kératineuses qui condensent plus intensément la chromatine du spermatozoïde que chez les Amphibiens ou les Poissons à fécondation externe possédant des histones dans le spermatozoïde. Dans de tels taxons, les plus grandes unités relativement constantes de similarité évolutive ("R-CLUES" en Anglais) ont une plus grande étendue phylogénique chez les Mammifères (par exemple, l'infraclasse Eutheria) que chez les Amphibiens (par exemple, le genre *Bufo*). L'évolution des PNBS apparaît saltatoire plutôt que continue. De ce fait, l'ampleur phylogénique des "R-CLUES" peut être utile pour la classification de taxons particuliers chez lesquels les PNBS sont proches des protamines ou proches des histones, parmi des genres particuliers d'Amphibiens (*Xenopus* versus *Silurana*) ou des familles ou sous-familles de Poissons (Salmoninae versus Sparidae). SUBIRANA & COLOM (1987), AUSIO *et al.* (1987), et CHIVA *et al.* (1991) ont proposé que les protamines chez les Mollusques aient évolué à partir d'histones H1 très riches en lysine. Cette hypothèse est confortée par la présence de PNBS proches des protamines avec des domaines résistants à la trypsine dans les spermatozoïdes des Cnidaires. La présence de PNBS chez les plantes inférieures pourrait être un cas d'évolution convergente. Les chromosomes hautement condensés du



Dinoflagellé Eucaryote *Cryptothecodinium cohnii* et les nucléoïdes très condensés de la Bactérie *Chlamydia trachomatis* ont tous deux des protéines basiques riches en lysine, respectivement similaires à des portions des histones H5 et H1. Ceci suggère l'existence de cas supplémentaires d'évolution convergente parmi les protéines basiques de la chromatine condensée, antérieurs à l'apparition du type primitif de spermatozoïde chez les Métazoaires.

Sperm basic proteins (SNBPs) that bind to DNA in animals and plants are highly diverse [12], in sharp contrast to the evolutionarily conservative nucleosomal histones that characterize all other cell types. SNBPs range from low molecular weight arginine-rich protamines in which these basic residues are clustered repeatedly, as in the sperm nucleus of the cartilaginous fish *Scyliorhinus canicula* [17], to replacement by a sperm-specific variant of histone H1, as in the frog *Rana ridibunda* [34]. In sperm of the goldfish *Carassius auratus* [40], the entire complement of histones is retained along with the nucleosomal organization of chromatin. Keratinous protamines with disulfide bonds also can be found in the sperm of *Scyliorhinus* [17]. Some protamine-like SNBPs show similarities to very lysine-rich histones, as in bivalves like the surf clam *Spisula solidissima* [7] and the mussel *Mytilus edulis* [8]. Other protamine-like SNBPs have intermediate compositions containing lysine and sometimes histidine along with arginine, as in the turtle *Chrysemys picta* [20], some frogs like *Ascaphus truei* [33] and *Bufo japonicus* [60], and in some bony fish, including sticklebacks like *Pungitius pungitius* [37]. Some crab species have no basic proteins at all in their sperm [12].

#### DISCUSSION

##### *Internal fertilization as a constraint on SNBP diversity*

Is this diversity of SNBP type due to randomness or adaptation? In BLOCH's classical paper he came down on the side of the former when he stated, "It is proposed that the variability (non-conservatism) of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus" [12, p. 107]. In the same paper, he also indicated that "... although a phylogenetic relationship is often apparent from the similarity of proteins within tightly defined taxonomic groups (e.g., the clupeids, or the eutheria), there seems to be no evolutionary trend. Most of the classes of sperm proteins are represented within most of the broad taxa." [12, p. 99]. In fact, analyses [30-32, 53] of the distribution of SNBPs in animals indicates (Fig. 1, left) that the mode of fertilization acts as a constraint on SNBP diversity, such that either protamines, keratinous protamines or protamine-like SNBPs are present in sperm of internally fertilizing taxa. Thus, sperm of the honey bee *Apis mellifera* [5] and the barnacle *Balanus nubilus* [21] are not exceptions to this rule, as thought by BLOCH [12], but appear to contain protamine-like SNBPs upon gel electrophoresis, while cytochemical analysis [53] indicates the presence of protamine-like SNBPs in sperm of the platyhelminth *Notoplana* and the nematode *Thelastoma periplaneticola*. The only exception to this rule known to date is the deep sea bony fish *Cataetx laticeps* (order Ophidiiformes, family Bythitidae). Recently, SAPERAS *et al.* (58) have discovered that internally fertilizing sperm of this species contain histones and an additional sperm-specific protein that is compositionally similar to erythrocyte H5 from grass carp. Perhaps this anomaly is a consequence of the fact that internally fertilizing deep sea fish such as brotulas have relatively short larval stages [41, p. 469], a reproductive strategy designed to enable a small number of young to settle in suitable habitats in their benthic environment. Such a progenetic tendency [39] in this viviparous fish might require the utilization of histone-like genes, rather than the more usual protamine-like genes to condense DNA, as the first sperm might have to be made after fewer cell divisions following fertilization in such a precociously maturing organism [35]. It may be possible to test this experimentally by examining the relationship between SNBP type and progenesis in the five species of bythids that are confined to shallower freshwater or weak brackish water environments [41, p. 226].



Internal fertilization as a constraint on SNBP diversity is most clearly observed in the tetrapods. In the subphylum Vertebrata (Fig. 1, right), internally fertilizing eutherian mammals possess keratinous protamines [10, 42], metatherians have non-keratinous P1-like SNBPs [63], birds contain protamines [19, 42] and reptiles have protamine-like SNBPs [20, 30]. In some frogs with external fertilization, like *Rana*, sperm-specific histones H1 and H2B are present in the nucleus. Here [50], sperm chromatin is not as tightly condensed as in amniote species with protamines or protamine-like SNBPs. Thus, there is a trend in the tetrapods from variability of SNBP type in externally fertilizing anurans to relative constancy of protamine type in internally fertilizing urodeles and amniotes [30, 31]. This can also be observed in cartilaginous fish [17, 30] and in bony fish [12, 30], with the sole exception of *Cataetyx laticeps* [58] noted above. In molluscs [18, 23], internal fertilization also constrains SNBP diversity in mesogastropods, neogastropods and cephalopods. Here protamine-like SNBPs or keratinous protamines are found.

#### *R-CLUES as phylogenetic measures of SNBP diversity.*

If we define a new term, “R-CLUES,” as the “relatively constant, largest units of evolutionary similarity [32], then amniotes have phylogenetically broader R-CLUES than externally fertilizing vertebrates like anurans and most bony fish by virtue of the constraint of internal fertilization. For example, Table 1 shows that similar P1 keratinous protamines occur throughout the *infra*class Eutheria, but similar protamine-like intermediate SNBPs are confined to particular *genera* of Australian frogs, like *Litoria* [33], and stickleback fish, such as *Gasterosteus* [37].

The acronym “R-CLUES” is also intended to denote the search for “clues” for “R,” the arginine content of SNBPs in particular taxa. Thus, a low arginine content of 4.5 mole percent in the lysine-rich protamine PL-IV of the mussel *Mytilus edulis* [8] is typical for R-CLUES at the family level (Mytilidae) in the class Bivalvia. In the genus *Bufo*, R-CLUES for these representatives of the order Anura are delineated by a much higher arginine composition, for example 42.3 mole percent, in *Bufo japonicus* protamine P1 [60].

Is internal fertilization the only constraint on SNBP diversity in animal sperm? Apparently not. As seen in Table 1, echinoderms are all external fertilizers. Constancy in the marine environment probably accounts for the relative constancy of SNBP type in this taxon. All of the echinoderms have sperm-specific histone H1 [44], with R-CLUES showing variation for different orders [30]. In externally fertilizing tunicates, the constant marine environment also appears to maintain the relative constancy of SNBPs. A protamine-like P1 SNBP that resembles H1 histone is the principle protein for all species studied thus far [22, 56], with some variation for R-CLUES for the genus *Styela*. Therefore, from R-CLUES indicated in Table 1, we see that internal fertilization is a particular kind of constraint for certain taxa on land, like amniotes, and in the marine environment, like mesogastropods, neogastropods, cephalopods and cartilaginous fish. I agree with Saperas [55, p. 330] that internal fertilization tends to fix the type of SNBP, insofar as the more specialized the biology of reproduction, the more such variation impacts negatively on SNBP function.

As can be seen in Fig. 1 and Table 1, the evolution of SNBPs appears to be saltatory rather than continuous, in the sense that these proteins can differ quite markedly between related taxa. In the case of eutherian mammals, P1 keratinous protamines are sufficiently similar to constitute a family of related proteins [17], yet they are amongst the most rapidly diverging polypeptides studied [47], evolving at rates close to that of fibrinopeptides. Nevertheless, these proteins can easily be distinguished from the protamines of metatherian mammals [63] and the arginine-rich protamines of birds [19, 42], both of which lack cysteine.



TABLE 1. — R-CLUES of representative animal taxa.

<sup>1</sup>, Classification modified from [11, 28, 41, 65]. <sup>2</sup>, See legend of Fig. 1 for definitions of P, KP, PL and H. H1 = sperm-specific histone H1, with higher arginine content than somatic histone H1. H2B = sperm-specific histone H2B. <sup>3</sup>, R-CLUES (relatively constant largest units of evolutionary similarity) are in italics. <sup>4</sup>, Int. = internal; Ext. = external. <sup>5</sup>, Ter. = terrestrial; Mar. = marine; FW = freshwater. <sup>6</sup>, P1, P2a, P2b, = keratinous protamines. <sup>7</sup>, Reference. <sup>8</sup>, Taxonomic ranking as follows: phylum > subphylum > class > infraclass > superorder > order > suborder > family > subfamily > genus [11, 28, 41]. <sup>9</sup>, *Silurana* = proposed genus [14] to contain former species *Xenopus tropicalis* (2n=20) and *X. epitropicalis* (2n=40). <sup>10</sup>, Some primitive urodele species are external fertilizers [30]. <sup>11</sup>, Some species are anadromous; i.e., marine, but breed in freshwater [37]. <sup>12</sup>, *Ascidia* is a facultative internal fertilizer [22] in this suborder. <sup>13</sup>, Includes SNBPs from 3 of the 15 living families of suborder Lacertilia (Sauria) but from only one family (Colubridae) of 15 in suborder Serpentes [65]. <sup>14</sup>, In order Carcharhiniformes. May also include SNBPs of *Squalus acanthias* [30], family Squalidae, order Squaliformes and R-CLUES may therefore be broader phylogenetically than indicated here. <sup>15</sup>, May also include SNBPs of *Sepia officinalis* [23] in order Sepoidea.

Taxon <sup>1</sup>	SNBP Type <sup>2</sup> , Reference	R-CLUES <sup>3</sup>	Fertilization <sup>4</sup>	Habitat <sup>5</sup>
Phylum Chordata				
Subphylum Vertebrata				
Class Mammalia	KP(P1) <sup>6</sup> [10, 42] <sup>7</sup>	<i>Infraclass</i> <sup>8</sup> Eutheria	Int.	Ter., Mar.
	KP (P1+P2a+P2b) <sup>6</sup> [47]	<i>Order</i> Primates	Int.	Ter.
	PL [63]	<i>Order</i> Marsupalia	Int.	Ter.
Class Aves	P [19, 42]	<i>Superorder</i> Paleognathae	Int.	Ter.
Class Reptilia	PL [30]	<i>Order</i> Squamata <sup>13</sup>	Int.	Ter.
	PL [20]	<i>Order</i> Testudines	Int.	Ter.
Class Amphibia	PL [30]	<i>Order</i> Caudata	Int., Ext. <sup>10</sup>	Ter.
	PL [14, 30]	<i>Genus</i> <i>Xenopus</i> + <i>Silurana</i> <sup>9</sup>	Ext.	Ter.
	PL [34, 60]	<i>Genus</i> <i>Bufo</i>	Ext.	Ter.
	PL [33]	<i>Genus</i> <i>Litoria</i>	Ext.	Ter.
	H1 [1, 34]	<i>Genus</i> <i>Rana</i>	Ext.	Ter.
Class Osteichthyes				
Division Teleostei	PL [37]	<i>Genus</i> <i>Gasterosteus</i>	Ext.	Mar., FW <sup>11</sup>
	P [42]	<i>Subfamily</i> Salmoninae	Ext.	Mar.
	H [58]	<i>Family</i> Sparidae	Ext.	Mar.
Class Chondrichthyes				
Subclass Elasmobranchii	KP, P [17, 43]	<i>Family</i> Scyliorhinidae <sup>14</sup>	Int.	Mar.
Subphylum Urochordata	PL (P1) [22]	<i>Suborder</i> Phlebobranchiata	Ext. <sup>12</sup>	Mar.
	PL (P1, P2) [22, 56]	<i>Genus</i> Styelidae	Ext.	Mar.
Phylum Echinodermata				
Class Holothuroidea	H1, $\phi$ o [45]	<i>Genus</i> <i>Holothuria</i>	Ext.	Mar.
Class Echinoidea	H1, H2B [30, 44]	<i>Order</i> Echinoidea	Ext.	Mar.
Subclass Asteroidea	H1 [44]	<i>Order</i> Forcipulata	Ext.	Mar.
Phylum Mollusca				
Class Polyplacophora	PL (P1, P2) [7, 23]	<i>Genus</i> <i>Mopalia</i>	Ext.	Mar.
Class Gastropoda				
Order Archaeogastropoda	PL (P2) [18, 23]	<i>Family</i> Trochidae	Ext.	Mar.
Order Patellogastropoda	PL (P1) [18, 23]	<i>Family</i> Lottiidae	Ext.	Mar.
Order Mesogastropoda	PL (P3) [18, 23]	<i>Family</i> Littorinidae	Int.	Mar.
Order Neogastropoda	PL (P3) [18, 23]	<i>Family</i> Nuccellidae	Int.	Mar.
Class Bivalvia				
Subclass Pteriomorphia	PL [23]	<i>Family</i> Mytilidae	Ext.	Mar.
Subclass Heterodonta	PL [23]	<i>Family</i> Tridacnidae	Ext.	Mar.
Class Cephalopoda	P [23]	<i>Order</i> Teuthodea <sup>15</sup>	Int.	Mar.



Amongst the vertebrates, the saltatory nature of SNBP evolution can best be seen in frogs and bony fish. Table 1 shows that different genera of frogs can have either histone-like SNBPs or arginine-rich protamine-like SNBPs, whereas some bony fish can show a similar alteration at the familial level (subfamily Salmoninae versus family Sparidae). This alteration appears to be of a sporadic nature [58]. Analysis of such R-CLUES may assist us in determining whether protamine  $\rightarrow$  histone or histone  $\rightarrow$  protamine transitions may have occurred in closely related taxa. The clearest example we have to date of the success of such an analysis is the ability of R-CLUES to distinguish between frogs of the genus *Xenopus* [61] and morphologically similar frogs of the genus *Silurana* [14]. In the polyploid genus *Xenopus*, there is a lineage of frogs with diploid chromosome numbers of 36  $\rightarrow$  72  $\rightarrow$  108 that includes *laevis* and another lineage with 20  $\rightarrow$  40 diploid chromosome numbers that includes *tropicalis* and *epitropicalis*. Only SNBP type can clearly distinguish these two lineages biochemically. Thus, R-CLUES can distinguish two separate genera, since the former lineage has intermediate type, protamine-like SNBPs, while the 20  $\rightarrow$  40 line contains only histones, with one additional spermatid/sperm-specific protein [38].

Recently, on the basis of morphological criteria, CANNATELLA & TRUEB [14] have split the genus *Xenopus* and have placed the discordant *tropicalis* and *epitropicalis* species into the genus *Silurana*, which they have resurrected from GRAY (1864). Thus, amongst the five genera of pipid frogs [65, p. 364], the two species of *Silurana* are more closely related to *Hymenochirus* and *Pipa* than to species of *Xenopus*. As we predicted the electrophoretic profile of *Silurana epitropicalis* SNBPs solely on the basis of chromosome number before doing the actual experiment [38], it appears that R-CLUES may be useful characters for systematic studies, along with more traditional analyses.

#### *Origins of SNBPs.*

What is the origin of the SNBP pattern in the deuterostomes? Recently, SAPERAS *et al.* [57] presented two models, both of which take into account the difference between histones present in echinoderm sperm and protamine-like SNBPs in urochordates and the cephalochordate *Branchiostoma floridae*. The lamprey *Petromyzon marinus* also has somatic-like histones in its sperm [57]. However, cartilaginous fish have protamines and keratinous protamines [17]. Alternation between protamines and histones can be seen in different families of bony fish and frogs, whereas urodeles and the amniotes have only protamines, protamine-like SNBPs or keratinous protamines.

From these data, SAPERAS *et al.* [57] concluded that both models would have histones at the base of the SNBP pattern in deuterostomes. However, examination of Fig. 1 (left) indicates that at the root of deuterostome phylogeny are taxa that show protamine-like SNBPs, either by cytochemical criteria, as in nematodes and platyhelminths [53], or by a combination of cytochemical and biochemical analysis, as in cnidaria [6, 53]. In the case of the sea anemone *Metridium senile*, AUSIO [4] has shown that the SNBP belongs to the PL-I type, related to H1 histones, with a peptide core that is trypsin resistant. It would seem, therefore, that another model could place protamine-like SNBPs at the base of deuterostome phylogeny, with reversions to histones occurring in particular taxa like echinoderms and agnatha. This might be due to the loss of protamine genes or gene expression in deuterostome evolution [57], or perhaps due to shifts in developmental timing, such that an earlier onset of spermiogenesis in particular taxa might require selection for histone rather than protamine gene expression [35]. Such an argument was made in the previous section for the lack of protamines in the internally fertilizing deep sea fish *Cataetys laticeps* [58]. Perhaps it might also apply to the histone-like SNBPs in sessile echinoderms evolved from motile ancestors [11, p. 838].

What might be the origins of protamine-like SNBPs in the lower metazoa? SUBIRANA & COLOM [59], AUSIO *et al.* [9] and CHIVA *et al.* [18] have proposed that protamines in molluscs

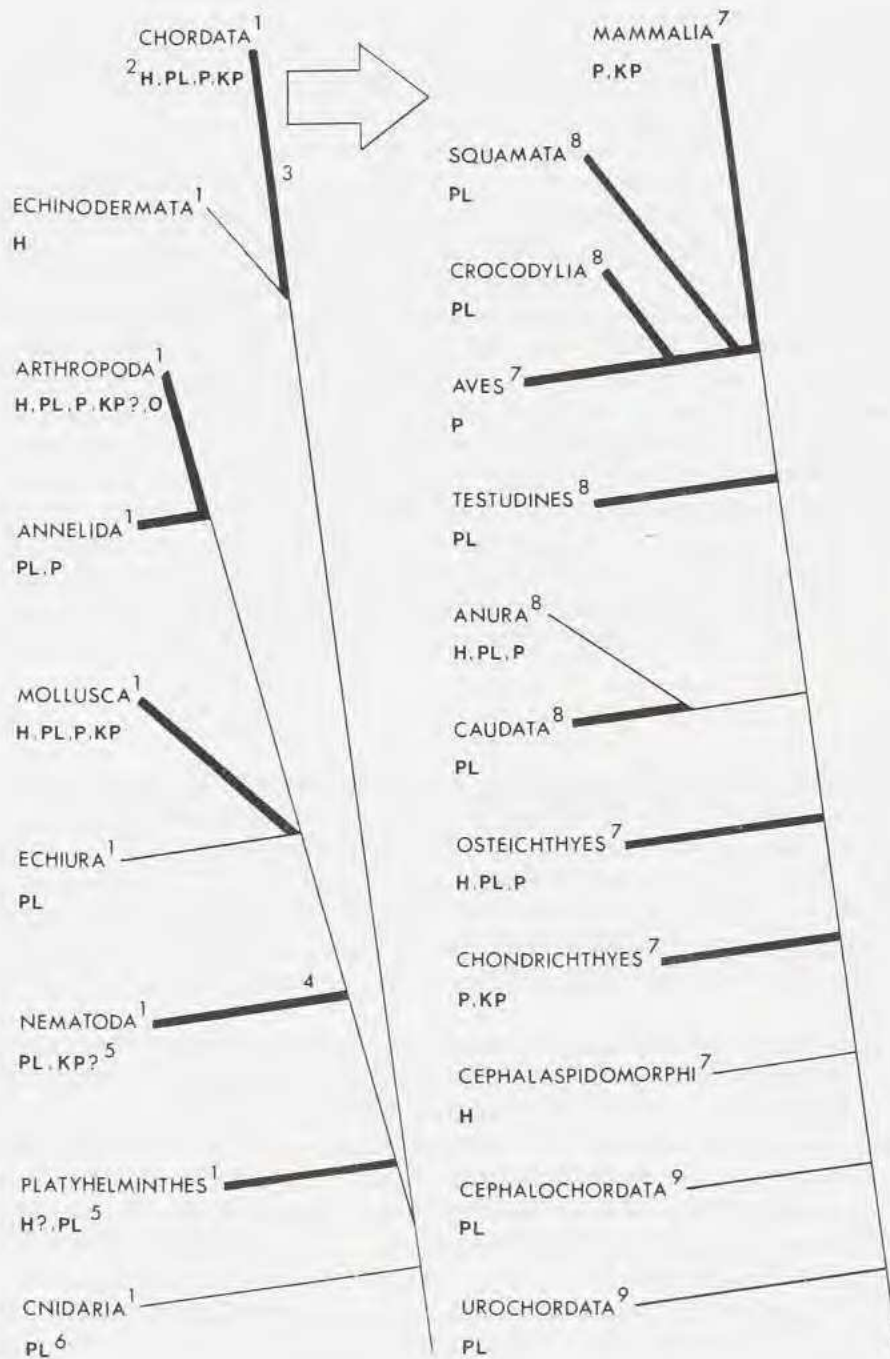


FIG. 1. — Diversity of SNBPs in animals. (Cladogram on left has been modified from [13, pp. 873, 882] and on right modified from [28, pp. 607, 628, 669, 684, 707, 736]. 1, Phylum; 2, SNBP type: H = somatic like-histones; PL = intermediate protamine-like SNBPs; P = protamines; KP = keratinous protamines; 0 = no SNBPs present in sperm nucleus; 3, Thick line indicates some or all species in this taxon are internally fertilizing; 4, Position of Nematoda is not indicated in [13, p. 882]; 5, SNBP type based on cytochemical data [12, 53] or 6, on electrophoretic and cytochemical data [6, 53]; 7, Class; 8, Order; 9, Subphylum.



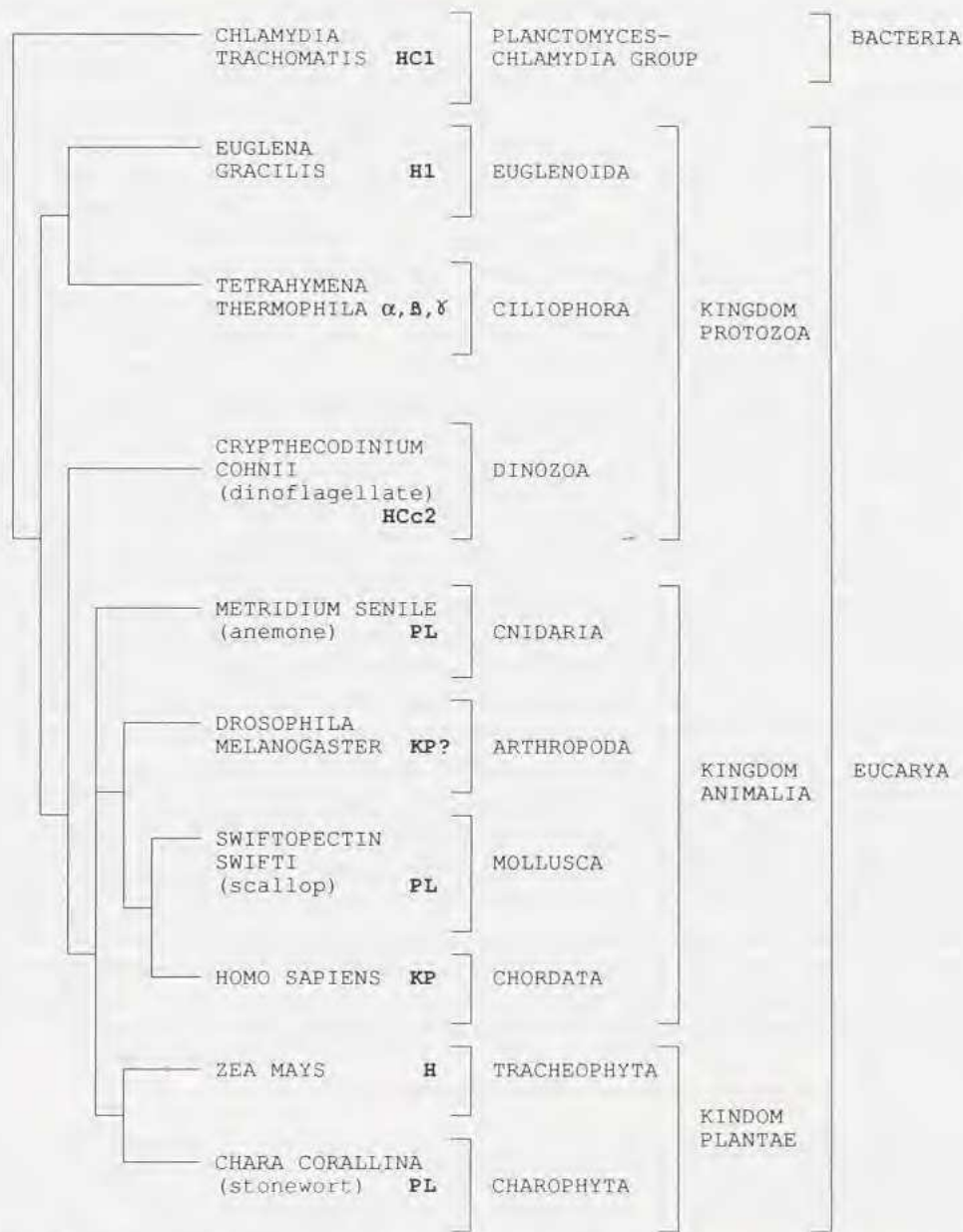


FIG. 2. — Possible outgroups of animal SNBPs amongst nuclear basic proteins of plant sperm and protists. Cladogram is modified from CAVALIER-SMITH's 18s rRNA phylogeny for 150 eukaryotes [16] and WOESE's 16s rRNA phylogeny for bacteria [64].

may have arisen from very lysine-rich H1 histones by the evolutionary route H1 histones → protamine-like SNBPs → protamines. The presence of PL-I basic proteins in cnidarians [4, 6] supports such an evolutionary pathway. Also, as can be seen in Fig. 2, plant SNBPs [51] provide an outgroup for patterns of SNBP diversity in animal phyla since they arose from different protistan ancestors than did animals. Therefore, the presence of protamine-like SNBPs in biflagellate, motile sperm of green algae, such as the stonewort *Chara corallina* [49], and in bryophytes, like the liverwort *Marchantia polymorpha* [48], along with the presence of H1-like histones in condensed nuclei of the nonmotile male gamete in a higher plant such as *Lilium*



*longiflorum* [62], indicates that the histone H1 → protamine-like SNBP transition may be a case of convergent evolution [24]. This attests to the importance of the connection between sperm motility and the condensation of sperm chromatin in the origin of SNBPs, a connection that is further emphasized if we consider the nature of the basic proteins in highly condensed nuclei of chromosomes in protists [46, p.10]. Thus, the eukaryotic dinoflagellate *Cryptothecodinium cohnii* [54], which has condensed chromosomes, lacks histones and nucleosomes but contains two lysine-rich base proteins, one of which, HCc2, shows 38% of residues identical with the 53 residues near the carboxyl terminus of the erythrocyte-specific histone H5 of duck. More primitive dinoflagellates have histones, whereas in *Noctiluca* [15], a transitional form, histones are present in the giant vegetative cells, but not in the small sexual swarmer stages. As CAVALIER-SMITH [15, p. 350] points out, "It could be that histone loss in the noctilucean swarmer is adaptive, analogous to the replacement of histones by protamines or other basic proteins in many animal sperm to allow more compact sperm nuclei." The histone H1 in nuclei with condensed chromatin in *Euglena gracilis* [29] is unique and in the condensed, transcriptionally inactive, germ-line micronuclei of *Tetrahymena thermophila*, [2], proteolytic processing of histone H1 gives rise to three H1-like polypeptides,  $\alpha$ ,  $\beta$  and  $\gamma$ . GOROVSKY [26] notes that "like some other transcriptionally inert nuclei such as nucleated red blood cells [25] and some histone containing sperm [1], micronuclei have different linker-associated (H1-type) histones than their transcriptionally active counterparts." RAIKOV [46, p. 71] calls the micronuclei of many Ciliophora "spermal" as they "are filled with compact chromatin, just as the nuclei of spermatozoa are." In this regard it is interesting to note that the highly condensed nucleoid in the elementary body of the prokaryotic bacterium *Chlamydia trachomatis* [27] contains a basic protein, Hc1, that shows 34.9% identity in a 106-amino acid overlap with histone H1 of the sea urchin *Lytechinus pictus*. This is highly unusual for bacteria, however, where the nucleoid is normally not so highly condensed [52] but may none the less contain some H1-like basic proteins as in *Pseudomonas aeruginosa* [36], or protamine-like basic proteins as in *Escherichia coli* [3].

From the viewpoint of SNBP types in lower metazoans, there is a sense of déjà vu with regard to the role of H1-like histone and related basic proteins in condensing chromatin in some protists and a bacterium. Perhaps it is the common need for tight packing of chromatin in motile sperm of animals and lower plants and in the condensed nuclei or chromosomes of motile protists that has given rise, probably by convergent evolution, to some common features within the diverse spectrum of basic proteins binding to DNA in these different organisms.

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